Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia

(neurogenesis/subependymal zone/brain repair/stem cells)

CARLOS LOIS AND ARTURO ALVAREZ-BUYLLA

The Rockefeller University, 1230 York Avenue, New York, NY 10021

ABSTRACT   Subventricular zone (SVZ) cells proliferate spontaneously *in vivo* in the telencephalon of adult mammals. Several studies suggest that SVZ cells do not differentiate after mitosis into neurons or glia but die. In the present work, we show that SVZ cells labeled in the brains of adult mice with [³H]thymidine differentiate directly into neurons and glia in explant cultures. *In vitro* labeling with [³H]thymidine shows that 98% of the neurons that differentiate from the SVZ explants are derived from precursor cells that underwent their last division *in vivo*. This report identifies the SVZ cells as neuronal precursors in an adult mammalian brain.

Neurons are born in the ventricular and subventricular zone (SVZ) of the developing central nervous system. In mammals, this is a process that ends before or soon after birth (1, 2). Adult neurogenesis in the mammalian brain has been described only in the dentate gyrus of the hippocampus and olfactory bulb (3–6). However, in these cases newly generated neurons are not born in the ventricular zone or SVZ but are thought to be derived from precursor cells located within these regions (3–6). In contrast, the brain of the adult bird retains the ability to generate neurons in the ventricular zone of the forebrain (7, 8). Interestingly, the brains of adult mouse (9), rat (10), dog (11), and monkey (12) retain a population of proliferating cells in the SVZ. These proliferating cells are located in the walls of the lateral ventricle in a region where neurons and glia are born during late stages of embryonic development (1). Recent experiments suggest that the progeny of SVZ cells in adult mice do not differentiate into neurons or glia *in vivo*; instead, it has been hypothesized that one daughter cell dies while the other one reenters mitosis (13). Recent works show that cells dissociated from adult mouse brain can be stimulated to proliferate *in vitro* when epidermal growth factor (EGF) (14) or basic fibroblast growth factor (bFGF) (15) is added to the culture medium, and under appropriate conditions the progeny of these dividing cells differentiate into neurons and glia. In the present work, we label cells in the SVZ of adult mice with [³H]thymidine and show that these cells, which divide *in vivo*, differentiate *in vitro* into neurons and glia. These experiments indicate that the SVZ of the adult mammalian brain retains the potential to generate new neurons.

MATERIALS AND METHODS

[³H]Thymidine Labeling *in Vivo*. Adult male mice (CD-1; 3–8 months old) received 5 i.p. injections of 50 μCi (6.7 Ci/mmol; 1 Ci = 37 GBq; NEN) of [³H]thymidine every 12 hr.

Histology. Mice (*n* = 2) were deeply anesthetized with sodium pentobarbital (Nembutal) (6 hr after the last [³H]thy-

midine injection) and perfused with 3.7% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.3), and the brains were processed for autoradiography as described (8). Sections were mapped to determine position, number, and phenotype of labeled cells.

SVZ Explant Cultures. Mice (*n* = 5) received [³H]thymidine *in vivo* as described above and were sacrificed by cervical dislocation 6 hr after the last injection. This time was chosen so that no [³H]thymidine would be present in the extracellular fluids (16). The SVZ was dissected from a frontal slice (≈2 mm thick) extending between the crossing of the anterior commissure and the rostral opening of the third ventricle. A strip of tissue (≈100 μm wide; 2–4 mm long) was cut along the lateral wall of the lateral ventricle under the corpus callosum down to the ventral tip of the lateral ventricle (Fig. 1). Tissue was minced with scalpels into pieces of ≈0.15 mm. Explants were cultured onto 35-mm dishes coated with poly-(D-lysine) (50 μg per 100 mm²) containing 500 μl of culture medium. Each animal yielded 100–120 explants that were plated at a density of 30 explants per dish. The medium composition was as follows: 50% Dulbecco's modified Eagle's medium (GIBCO), 25% Earle's balanced salt solution (GIBCO), 25% donor horse serum (JRH Biosciences, Lenexa, KS), 1 mM glutamine (GIBCO), penicillin (50 units/ml), streptomycin (50 μg/ml), amphotericin (0.125 μg/ml) (GIBCO), and glucose (6 mg/ml) (Sigma). Explants were cultured at 36°C in a 7% CO₂/93% air atmosphere. Twenty-four hours after plating, 200 μl of medium was added to each of the dishes, and after 48 hr *in vitro* all the medium was replaced with 700 μl of fresh medium. The cultures were fixed after 6 days *in vitro* (DIV).

Cortex and Striatum Cultures. Mice (*n* = 5) received [³H]thymidine *in vivo* as described above. Explants from striatum and cortex were obtained from the regions indicated in Fig. 1 and were processed as described for the SVZ.

***In Vitro* [³H]Thymidine Labeling.** Mice (*n* = 2) were killed and the SVZ was dissected as described above. Culture medium (as described above) was supplemented with [³H]thymidine (1 μCi/ml) for labeling dividing cells. Cultures were exposed to [³H]thymidine either for the first 2 DIV or during the last 4 DIV. Similar results were obtained irrespective of the time when [³H]thymidine was present and data were pooled from these two groups. [³H]Thymidine is metabolically active to label the dividing cells *in vitro* during periods longer than 6 days (16).

Immunocytochemistry and Autoradiography of Cultures. After 6 DIV, explant cultures were washed for 5 min with Dulbecco's phosphate-buffered saline (DPBS) supplemented with CaCl₂ (0.1 g/liter) fixed for 10 min with 3.7% paraformaldehyde in PBS (pH 7.3), and washed three times with PBS

Abbreviations: SVZ, subventricular zone; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; DIV, days *in vitro*; BSA, bovine serum albumin; MAP-2, microtubule-associated protein 2; NF, neurofilament; NSE, neuron-specific enolase; GFAP, glial fibrillary acidic protein.
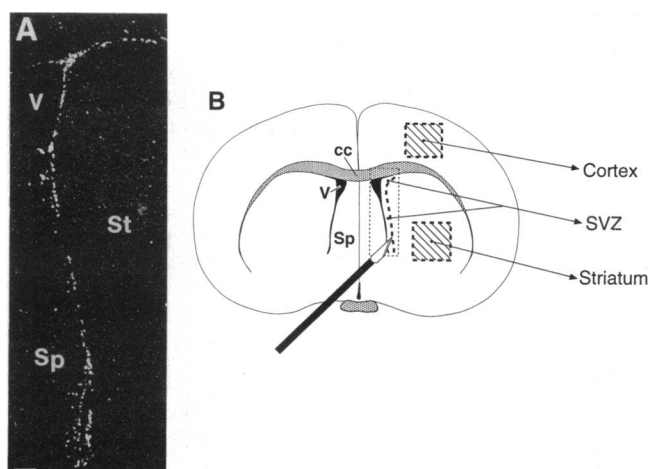
FIG. 1. (A) Dark-field photomicrograph of a frontal section (6 μm thick) of adult mouse SVZ. After [³H]thymidine treatment *in vivo* (as described below), most of the labeled cells are concentrated in the SVZ (white spots are [³H]thymidine-labeled nuclei). V, lateral ventricle; Sp, septum; St, striatum. (Bar = 100 μm.) (B) Schematic diagram of a frontal section of mouse brain showing the dissected regions (boldface dotted lines). Dotted rectangle corresponds to the area shown in A. cc, Corpus callosum.

(5 min each). Cells were permeabilized with 0.25% Triton X-100 in PBS for 5 min and subsequently incubated with a solution containing 10% (wt/vol) bovine serum albumin (BSA) (Sigma). After 20 min, this solution was removed and the explants were incubated for 24 hr at 4°C with mouse monoclonal antibodies against microtubule-associated protein 2 (MAP-2) (Sigma; M4403) (dilution, 1:500), 200-kDa neurofilament (NF) (Sigma; N5389) (dilution, 1:100), rabbit polyclonal antiserum against neuron-specific enolase (NSE) (Polysciences; 16625) (dilution, 1:3000), and glial fibrillary acidic protein (GFAP) (a gift of L. Eng, Stanford University) (dilution, 1:100). These primary antibodies were diluted in a solution containing 3% BSA in PBS. Tissue culture dishes were rinsed three times (5 min each) with PBS and incubated with biotinylated horse monoclonal antibody against mouse IgG (dilution, 1:200 in PBS/3% BSA) for 1 hr at room temperature. After three washes with PBS (5 min each), the explants were incubated with an avidin-biotin solution (Vectastain *Elite*, Vector Laboratories) (1 drop of A reagent plus 1 drop of B reagent diluted in 5 ml of DPBS) for 30 min at room temperature. The dishes were washed three times with PBS (5 min each) and incubated for 4 min in a solution containing 0.2 mg of diaminobenzidine per ml and 0.01% H_2O_2 in PBS. Omission of the primary antibody resulted in no detectable staining. Culture dishes were covered with autoradiographic emulsion (Kodak NTB2) and incubated at 4°C for 15 days. Autoradiograms were developed in D19 (Kodak) at 17°C.

RESULTS

Proliferating cells in the adult mouse brain, as revealed by [³H]thymidine labeling (16, 17), were restricted almost completely (>98% of all labeled cells per section) to the walls of the lateral ventricle in the SVZ (Fig. 1). [³H]Thymidine-labeled cells outside of the SVZ had the morphology of glial or endothelial cells.

To test the potential fates of these dividing cells, the SVZ was isolated and cultured. Explants of the SVZ attached to the substrate within several hours after plating. During the first 2 DIV, few cells migrated out of the explant. By 6 DIV, the explants had generated an outgrowth consisting of flat glial cells (positive to GFAP) and birefringent cells growing

on top of the glial monolayer (Fig. 2). The birefringent cells had one or more processes that grew longer with time (Fig. 2). Birefringent multipolar cells were more frequent after several DIV. These process-bearing cells were neurons as defined by positive immunostaining with antibodies specific for MAP-2, NF, and NSE (18, 19) and absence of staining with antibodies against GFAP.

Explants stained with neuron-specific antibodies were processed for autoradiography to detect the presence of [³H]thymidine in the cell nuclei (Fig. 1). Microscopic examination of culture dishes revealed that the majority of neurons (84.38%) in the outgrowth surrounding an explant were labeled with [³H]thymidine (Table 1). Many of the flat glial cells in the outgrowth were also [³H]thymidine positive. The overall ratio of [³H]thymidine-labeled glia to neurons was 1:1, but individual explants displayed significant variations from this ratio. These results indicate that cells that were proliferating in the adult SVZ *in vivo* had the potential for generating neurons and glial cells. However, we do not know whether individual precursor cells from the SVZ can generate both cell types.

Control explants obtained from cortex or striatum excluding the SVZ were cultured and processed in the same way as the SVZ explants. No [³H]thymidine-labeled cells could be detected in any of these control experiments. In contrast to
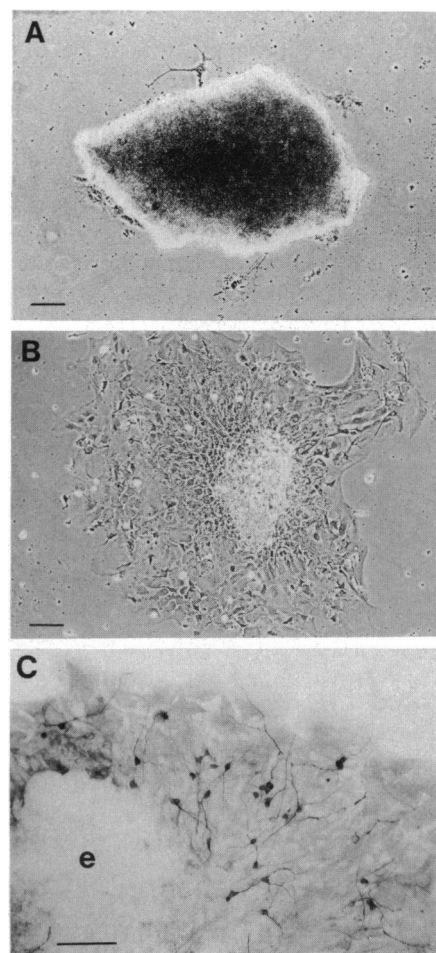

FIG. 2. Phase-contrast photomicrographs of live explants from striatum (A) and SVZ (B) after 5 DIV. SVZ explant generates an extensive outgrowth while a typical explant from striatum generates no outgrowth. (C) Outgrowth from a SVZ explant after fixation and immunocytochemical staining with MAP-2 monoclonal antibody. Neurons migrate throughout the outgrowth, differentiate, and extend processes on top of the glial monolayer. Position of explant that detached during staining is indicated by an e. (Bars = 100 μm.)

the sequence of events observed in SVZ explants, cortical and striatal explants never generated an outgrowth area (Fig. 2). A small number of glial cells (one to three per explant) emerged from some of the control explants and remained in the immediate vicinity of the explants, but these cells were negative to [³H]thymidine. These results indicate that in our culture conditions the SVZ tissue sampled, but not cortex or striatum, was able to generate neurons and glial cells.

To test whether the neuronal precursor cells from the SVZ continued to divide *in vitro*, explants were exposed to [³H]thymidine in culture (Fig. 3 and Table 1). A large number of the glial cells throughout the outgrowth were heavily labeled with autoradiographic grains over their nuclei when [³H]thymidine was present in the culture medium. This indicates that glial cells continue to divide *in vitro*. In contrast, in this same experiment, 17 neurons of 897 neurons counted (1.93%) were labeled with [³H]thymidine. The average number of neurons per explant was similar whether the culture medium contained or lacked [³H]thymidine, indicating that the presence of [³H]thymidine *in vitro* did not influence the differentiation or survival of neurons. These results indicate that the vast majority of neurons differentiating in culture were derived from precursor cells that underwent their last division *in vivo*.

DISCUSSION

The results presented here suggest that SVZ cells that divide spontaneously *in vivo* give rise to cells that differentiate into neurons or glia in culture. Recently, it has been reported that cells isolated from adult mouse brains can be stimulated to proliferate *in vitro* when EGF or bFGF is added to the culture medium, and under appropriate conditions the progeny of these dividing cells differentiate into neurons and glia (14, 15). In these previous studies, the brain was dissociated into single cells. This procedure did not allow characterizing the identity and location of the growth factor-responsive cells. In the present work, we labeled the SVZ cells *in vivo* and show that the daughter cells can become neurons *in vitro*. The SVZ may be the source of the cells that are sensitive to EGF and

Table 1. [³H]Thymidine-labeled cells in explant cultures

	SVZ		Cortex	Striatum
	[³H]-Thymidine *in vivo*	[³H]-Thymidine *in vitro*	[³H]-thymidine *in vivo*	[³H]-thymidine *in vivo*
Cultured explants	≈600	≈200	≈600	≈600
Productive explants	≈15%	≈15%	≈2%	≈2%
Mapped explants	57	16	11	15
Labeled glia	2789	1776	0	0
Labeled neurons	2576	17	0	0
Total neurons	3042	897	0	0
% neurons labeled	84.38%	1.93%	—	—

Explants were mapped under high-magnification objective (×63) with the aid of a computer-based mapping microscope. Cells were considered to be [³H]thymidine labeled when their nuclei were overlaid by five or more silver grains (>20 times background). Neurons were identified by MAP-2-, NSE-, or NF-positive staining. Numbers refer to total number of cells of the different categories in all the explants mapped.

bFGF in the *in vitro* conditions reported by other authors (14, 15), but this is still not known. Previous reports could be dealing with a completely different set of cells, which are normally dormant in the brain and are induced to proliferate when exposed to mitogens *in vitro*. Here we show that the progeny of SVZ stem cells differentiate in culture directly into neurons and glia after having completed their last division in the brain. SVZ cells do not have to divide *in vitro* in order to generate neurons. In sum, we show that the SVZ contains a population of neural stem cells that divide in adult mammalian brains and have the potential to differentiate directly into neurons and glia.

In our culture conditions, glial cells continued to divide *in vitro* but most neuronal precursors did not. We also observed that after [³H]thymidine labeling of the SVZ cells *in vivo*, approximately an equal number of labeled neurons and labeled glial cells appeared in the cultures after 6 DIV. These observations suggest that the labeled SVZ cells initially
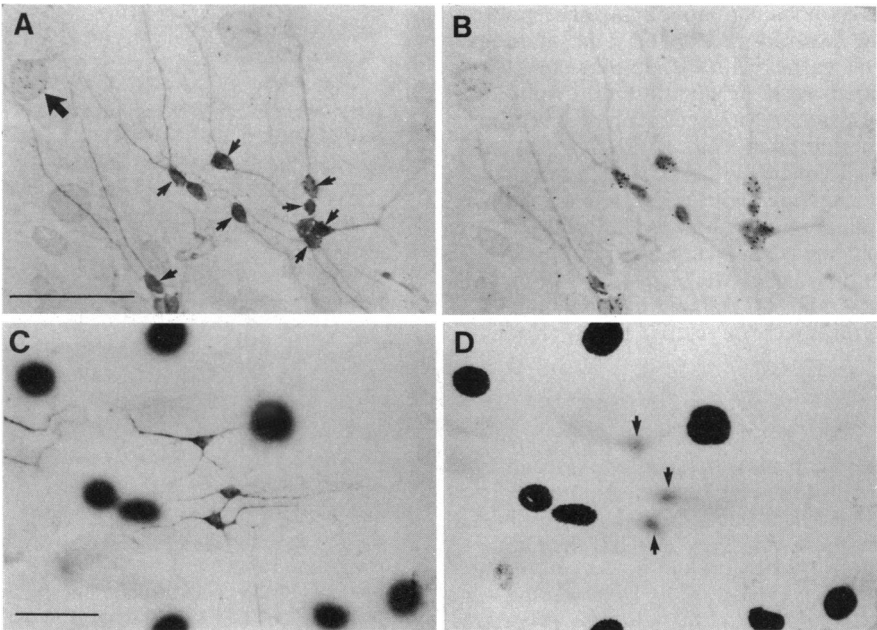

FIG. 3. (*A* and *C*) Photomicrographs show cells in focus. (*B* and *D*) Photomicrographs show autoradiographic grains in focus overlying the same cells shown in *A* and *C*. Most of the neurons (identified by MAP-2 staining; small arrows in *A*) are labeled by [³H]thymidine treatment *in vivo* as revealed by autoradiographic grains overlaying their nuclei (*B*). Glial cells are also labeled by [³H]thymidine treatment *in vivo* (large arrow in *A*). *In vitro* treatment with [³H]thymidine results in many heavily labeled glial cells that appear as large clusters of autoradiographic grains (*D*), but neurons (*C*) are not labeled (arrows in *D*). (Bars = 50 μm.)

generated more neurons than glial cells. However, other interpretations are also possible (e.g., the glial cells may have diluted the label after several rounds of proliferation), and it is difficult at this point to explain the ratio of neurons to glia that we showed arose from dividing SVZ cells. It will be of interest to determine the lineage dynamics of differentiation of adult SVZ cells.

In adult birds, new neurons continue to be generated in proliferating regions in the lateral wall of the lateral ventricle, a region topographically equivalent to the SVZ of mammals (8, 20). Explant cultures derived from the adult avian ventricular zone also generated neurons *in vitro* from precursors that divided *in vivo* (21). However, in adult birds young neurons migrate away from their birth site throughout the telencephalon to differentiate into neurons *in vivo* (20). These new neurons integrate into functional circuits in the brain (22). It has been hypothesized that neurogenesis in adult birds may be used as a form of plasticity for learning (23).

In the early postnatal period SVZ cells of mammals generate glial cells (1). It has been speculated that some SVZ cells may serve as a source of glial cells in adulthood (see ref. 1, p. 427). However, there is no evidence that the SVZ in adult mice generates neurons or glia *in vivo*. Instead, several lines of evidence indicate that the SVZ cells die *in vivo* after proliferation. There is a rapid decrease in the number of [^{3}H]thymidine or bromodeoxyuridine-labeled SVZ cells 3 days after injection (9, 13) and many pyknotic nuclei can be found in the SVZ (9). Moreover, recent experiments in which proliferating SVZ cells were labeled with recombinant retroviruses show that clones of retrovirus-infected cells contained only 1 or 2 cells and remain in the SVZ regardless of the survival time after infection (13). Although some retrovirally infected cells may fail to express the marker (see ref. 24), together these experiments (9, 13) are consistent with the interpretation that in the adult rodent brain death is the fate of half of the postmitotic SVZ cells.

We have shown that SVZ cells that divide in the brain spontaneously can differentiate into neurons under culture conditions. Perhaps dividing SVZ cells are retained as a source of neurons or glial cells for unknown targets in the brain or during environmental demands still to be identified (25). Alternatively, stem cell proliferation in the SVZ may have been retained as an evolutionary relic without function; neurogenesis in adult mammals may have been discontinued as part of an evolutionary process to ensure the stability of circuits and perhaps memory (2). Understanding the cellular and molecular mechanisms that control the proliferation and fate of SVZ cells of adult mammals may lead to a further understanding of the conditions that regulate these processes and enable induction of neurogenesis in adult mammals.

1. Jacobson, M. (1991) in *Developmental Neurobiology* (Plenum, New York), pp. 41–93.
2. Rakic, P. (1985) *Science* **227**, 1054–1056.
3. Bayer, S. A. (1985) *Ann. N.Y. Acad. Sci.* **457**, 163–172.
4. Altman, J. & Das, G. D. (1965) *J. Comp. Neurol.* **124**, 319–336.
5. Bayer, S. A., Yackel, J. W. & Puri, P. S. (1982) *Science* **216**, 890–892.
6. Kaplan, M. S. & Hinds, J. W. (1977) *Science* **197**, 1092–1094.
7. Goldman, S. A. & Nottebohm, F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2390–2394.
8. Alvarez-Buylla, A., Theelen, M. & Nottebohm, F. (1990) *Neuron* **5**, 101–109.
9. Smart, I. (1961) *J. Comp. Neurol.* **116**, 325–348.
10. Privat, A. & Leblond, C. P. (1972) *J. Comp. Neurol.* **146**, 277–302.
11. Blakemore, W. F. & Jolly, R. D. (1972) *J. Neurocytol.* **1**, 69–84.
12. McDermott, K. W. & Lantos, P. L. (1991) *J. Anat.* **178**, 45–63.
13. Morshead, C. M. & Van der Kooy, D. (1992) *J. Neurosci.* **12**, 249–256.
14. Reynolds, B. A. & Weiss, S. (1992) *Science* **255**, 1707–1710.
15. Richards, L. J., Kilpatrick, T. J. & Bartlett, P. F. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8591–8595.
16. Cleaver, J. E. (1967) in *Thymidine Metabolism and Cell Kinetics* (North–Holland, Amsterdam).
17. Sidman, R. L. (1970) in *Contemporary Research in Neuroanatomy*, eds. Nauta, W. J. H. & Ebbesson, S. O. E. (Springer, New York), pp. 252–274.
18. Fields, K. L. (1985) in *Cell Culture in the Neurosciences*, eds. Bottenstein, J. E. & Sato, G. (Plenum, New York), pp. 45–93.
19. Raff, M. C., Abney, E. R., Cohen, J., Lindsay, R. & Noble, M. (1979) *Brain Res.* **174**, 283–308.
20. Alvarez-Buylla, A. & Nottebohm, F. (1988) *Nature (London)* **335**, 353–354.
21. Goldman, S. A. (1990) *J. Neurosci.* **10**, 2931–2939.
22. Paton, J. A. & Nottebohm, F. (1984) *Science* **225**, 1046–1048.
23. Alvarez-Buylla, A., Kirn, J. R. & Nottebohm, F. (1990) *Science* **249**, 1444–1446.
24. Halliday, A. L. & Cepko, C. L. (1992) *Neuron* **9**, 15–26.
25. Altman, J. (1967) in *The Neurosciences*, eds. Quarton, G. C., Melnechuck, T. & Schmitt, F. O. (Rockefeller Univ. Press, New York), pp. 723–743.